Low dose streptozotocin causes stimulation of the immune system and of anti-islet cytotoxicity in mice

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SUMMARY

Multiple low doses of streptozotocin are known to induce immune-mediated insulin deficient diabetes and depression of immune reactivity. We show here that immune depression by streptozotocin is not general but that some parts of the immune system are stimulated. Spleen cells from streptozotocin-treated mice showed enhanced cytotoxicity against syngeneic islet cells and various tumour cells including insulinoma cells. Several cell types served as effector cells, including macrophages, asialo GM1$^+$ and Lyt-2$^+$ lymphocytes. The increased cytotoxic activity towards islet cells was mostly due to macrophages and to non-asialo GM1$^+$ and non-Lyt-2$^+$ lymphocytes. A higher activation state of macrophages in low dose streptozotocin-treated mice was demonstrated by measurements of superoxide anion release. We conclude that multiple low doses of streptozotocin stimulate 'natural cytotoxicity', i.e. the non-MHC restricted cytotoxic activity of macrophages, T cells and natural killer lymphocytes.

Keywords streptozotocin autoimmune diabetes islet cytotoxicity immunotoxicology interleukin 2

INTRODUCTION

Multiple administrations of low doses of streptozotocin have been shown to trigger chronic inflammation of pancreatic islets with subsequent hypoinsulinaemia and hyperglycaemia in male mice of several susceptible strains (Like & Rossini, 1976; Kolb, 1987). Experimental suppression of T-lymphocytes as well as the modulation of macrophage activity inhibits diabetes development (Rossini *et al.*, 1978; Oschilewski *et al.*, 1986). Depletion of either L3T4 (CD4) positive or of Lyt-2 (CD8) positive T cells suffices to impair progression of the disease (Kantwerk *et al.*, 1987; Herold, Montag & Fitch, 1987; Dayer-Metroz *et al.*, 1988). Despite this evidence of an immune nature of diabetes development, a definite proof of a role for autoimmunity could not be provided in adoptive transfer or islet transplantation experiments (Kolb, 1987; Wilson & Leiter, 1990).

Streptozotocin has been shown to damage not only islets but also many other tissues including lymphoid organs (Kolb, 1987). In general, depression of immune reactivity has been reported after high or multiple low doses of streptozotocin (Mahmoud *et al.*, 1976; Nevalainen & Hoftiezer, 1977; Nichols *et al.*, 1979; Ptak, Rewicka & Kollat, 1980; Ishibashi *et al.*, 1980; Nichols, Vann & Spellman, 1981; Busby & Rodman, 1983; Itoh *et al.*, 1984; Gaulton, Schwartz & Eardley, 1985). Since low dose

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streptozotocin-induced diabetes is immune mediated, a general depression of immune reactivity would be surprising. We demonstrate here that some arms of immunity are stimulated after low doses of streptozotocin.

MATERIALS AND METHODS

Low dose streptozotocin animal model
Hyperglycaemia was induced in 8-weeks-old male C57BL/6J mice from Bomholtgard, Ry, Denmark by injecting streptozotocin (Sz; Boehringer, Mannheim, Germany) (40 mg/kg body weight) intraperitoneally on five consecutive days (d0–d4). Control groups of sham-treated mice received injections of equivalent volumes of sodium-citrate buffer (Kantwerk *et al.*, 1987). Blood glucose was measured by the hexokinase method (Glucoquant, Boehringer).

Cytotoxicity assay
Enrichment of T/natural killer (NK) lymphocytes was performed by nylon wool passage of spleen cell suspensions. Depletion of a specific lymphocyte subset was performed by incubation of 5×10^6 spleen cells in 1 ml RPMI 1640 (2% fetal calf serum (FCS)) with specific antibodies (YTS 169.4–anti-Lyt-2, kind gift of Drs S. B. Cobbold and H. Waldmann, University of Cambridge, UK (Cobbold *et al.*, 1984); anti-asialo GM1, Wako Chemicals, Neuss, Germany) on ice for 30 min and subsequent incubation with low-tox-M-rabbit complement

(Camon, Wiesbaden, Germany). Subset-specific MoAbs were also used for pheno-typing cell populations by immunocytochemical staining with peroxidase as described previously (Kantwerk *et al.*, 1987). MoAbs to L3T4 (YTS 191.1.2) and to Thy-1 (YTS 154.7) were from Drs Cobbold and Waldmann (Cobbold *et al.*, 1984); anti-M1/70–15 and anti-mouse immunoglobulin were purchased from Camon. Macrophages were enriched > 90% by adherence of spleen cells for 1–2 h (37°C, 5% CO_2) on FCS-coated plastic dishes. Silica particles (0·5–5 μm) (kind gift of Steinkohlen-Bergbau-Verein, Essen, Germany) were added to some cultures (1 mg/ml) to inhibit macrophage function. For some experiments effector cells were stimulated with 100 U/ml human recombinant IL-2 (kind gift of Dr M. Wrann, Sandoz, Vienna, Austria) for 24 h before co-cultivation with target cells.

Rat insulinoma cell line RINm5F (kind gift of Dr T. Lander, Münich, Germany), YAC-1 mouse lymphoma cell line (NK-sensitive, kind gift of Dr M. Malter, Heidelberg, Germany) and EL-4 mouse thymoma cell line (LAK-sensitive, kind gift of Dr A. Reske-Kunz, Mainz, Germany) were maintained in modified RPMI 1640 medium with 10% FCS. RIN-cell monolayers were dissociated into single cells by trypsin before use as target cells. Pancreatic islets of untreated C57BL/6J mice were isolated by ductal injection of collagenase (Serva, Heidelberg, Germany, 0·37 U/mg, 3 mg/ml in Hank's Balanced Salt Solution (HBSS)) and subsequent centrifugation of the dispersed tissue on a Ficoll density gradient (Ficoll 400, Pharmacia, Freiburg, Germany) and dissociation of islets into single cells were performed as described previously (Appels *et al.*, 1989). Target cells (2 × 10⁵/ml) were labelled with ³H-leucine, 4–6 μCi/ml (specific activity 1 mCi/ml, Amersham-Buchler, Braunschweig, Germany) as described previously (Appels *et al.*, 1989).

Labelled target cells were resuspended in RPMI 1640 with 4 mM glucose and 10% FCS and seeded at 5000 cells in 100 μl/well in 96-well V-bottom (non-adherent effector cells) or round-bottom microculture plates (adherent effector cells) (Becton Dickinson, Heidelberg, Germany). Effector cells were added at 5–40 × 10⁴ cells in 100 μl/well in triplicate. After 16 h of co-incubation (5% CO_2, 37°C) the ³H content of 100 μl of supernatant was determined by liquid scintillation counting. Total ³H-leucine release was measured by adding 100 μl 10% Triton-X (Serva) to wells containing target cells. The total release was 11015±4446 ct/min (±s.e.m.) for YAC-1 cells, 21551±5008 ct/min (±s.e.m.) for EL-4 cells, 7960±1337 ct/min (±s.e.m.) for RINm5F, and 12499±1563 ct/min (±s.e.m.) for islet cells. The spontaneous release of ³H-leucine from the target cells in the absence of effector cells was 20–45% of the total ³H-leucine release after 16 h in all experiments. The cytotoxic activity of effector cells was calculated according to the formula:

Specific lysis (%) =

$$100 \times \frac{\text{(test ct/min} - \text{spontaneous ct/min)}}{\text{(maximal ct/min} - \text{spontaneous ct/min)}}.$$

Chemiluminescence assay

Plastic adherent spleen cells were prepared as described above. Of the cell suspension (2 × 10⁵/ml in RPMI 1640, 10% FCS) 1 ml was given into a minivial and incubated overnight (37°C, 5% CO_2). RPMI 1640 medium with non-adherent cells was removed, tubes with the remaining adherent cells were refilled with 500 μl Lucigenin (Sigma) 0·1 mM in Dulbecco's Minimal

Essential Medium (DMEM) (Boehringer). Oxidative metabolism was induced by adding zymosan (Sigma) particles (100 μg in 500 μl DMEM) or phorbol myristate acetate (PMA, Sigma; 12·5 μg in 500 μl DMEM). Chemiluminescence was measured for 10 s in a Biolumat 9500 (Berthold, Wildbad, Germany) at 37°C at various times over a period of 3–4 h. Background chemiluminescence was determined in vials with Lucigenin and DMEM only.

Cell proliferation assay

For mitogenic stimulation concanavalin A (Con A, Serva), phytohaemagglutinin (PHA; Wellcome, Beckenham, UK) and pokeweed mitogen (PWM, Sigma) was used at 1·25 μg/ml. Of the spleen cell suspensions (1 × 10⁶ cells/ml in RPMI 1640 with 2% freshly prepared normal mouse serum) 200 μl were given in each well of a 96-well flat-bottom microculture plate and incubated for 72 h (37°C, 5% CO_2) with or without mitogens. Per well 1 μCi ³H-TdR (spec. activity 1 mCi/ml, Amersham-Buchler) was added for the last 18 h. The cultures were harvested (Titertek cell harvester, Flow Laboratories, Meckenheim, Germany) and the ³H-TdR incorporation was measured by liquid scintillation counting. As determined by the Trypan blue exclusion test the viability of the spleen cells was 95–98% at the beginning and at the end of the proliferation assays.

Statistical analysis

Student's *t*-test (two-sided) and non-parametric correlation analysis according to Spearman were applied. The level of significance was set as $P < 0.05$.

RESULTS

Mononuclear spleen cels from low dose streptozotocin-treated mice showed enhanced cytotoxic activity against rat insulinoma (RIN) cells and syngeneic islet cells (Fig. 1). Significantly increased lysis ($P < 0.05$) was seen at all target:effector cell ratios tested. When YAC-1 cells or EL-4 tumour cells were used as targets, again significantly more cytotoxic activity was observed for splenocytes from low dose streptozotocin-treated mice ($P < 0.05$) (Fig. 1).

Identification of the cytotoxic effector cells was tried. Spleen cells were enriched for non-adherent T- and NK-lymphocytes by passage over a nylon wool column for the depletion of B-lymphocytes and macrophages (Table 1). The loss of adherent cells caused a reduction of the cytotoxic activity against YAC-1, RIN, and syngeneic islet cells (Fig. 2, column 1). The residual lytic activity by nylon wool-enriched T- and NK-cells was further characterized (Fig. 2, columns 2–6). Cytotoxicity towards YAC-1 cells could be stimulated by IL-2 treatment and inhibited by depletion of either asialo GM1 or Lyt-2⁺ lymphocytes. Lytic activity in the absence of Lyt-2⁺ cells was restored by IL-2.

Cytotoxicity towards RIN cells could also be stimulated by IL-2 treatment and inhibited only by the depletion of Lyt-2⁺ cells. Cytotoxicity towards islet cells by nylon wool-enriched lymphocytes was not stimulated by IL-2 and, surprisingly, not inhibited by depletion of Lyt-2 or asialo GM1⁺ cells.

Next, we analysed cytotoxicity against islet cells among adherent spleen cells. As shown in Fig. 3, adherent spleen cells lysed islet cells to a similar degree as did unfractionated spleen
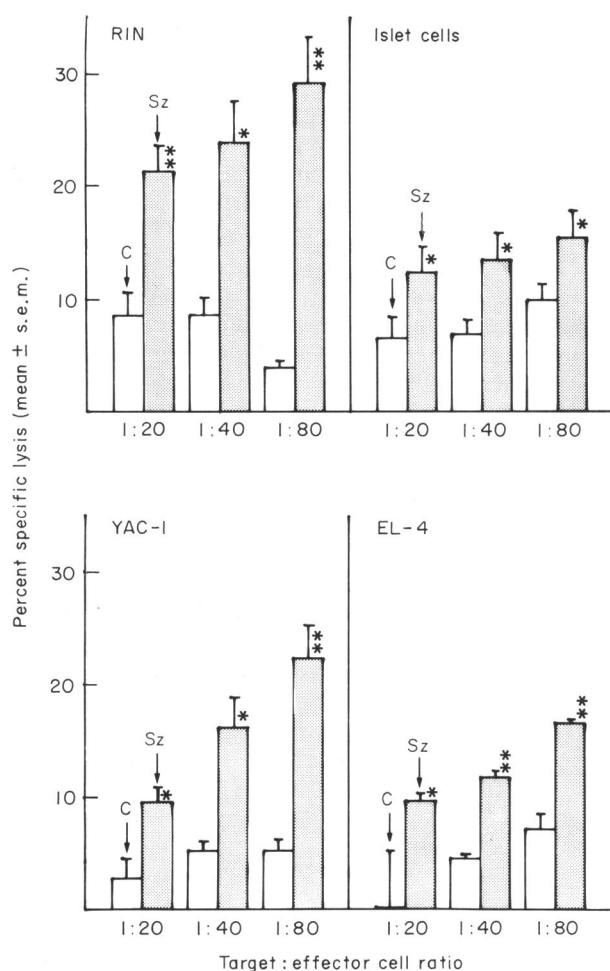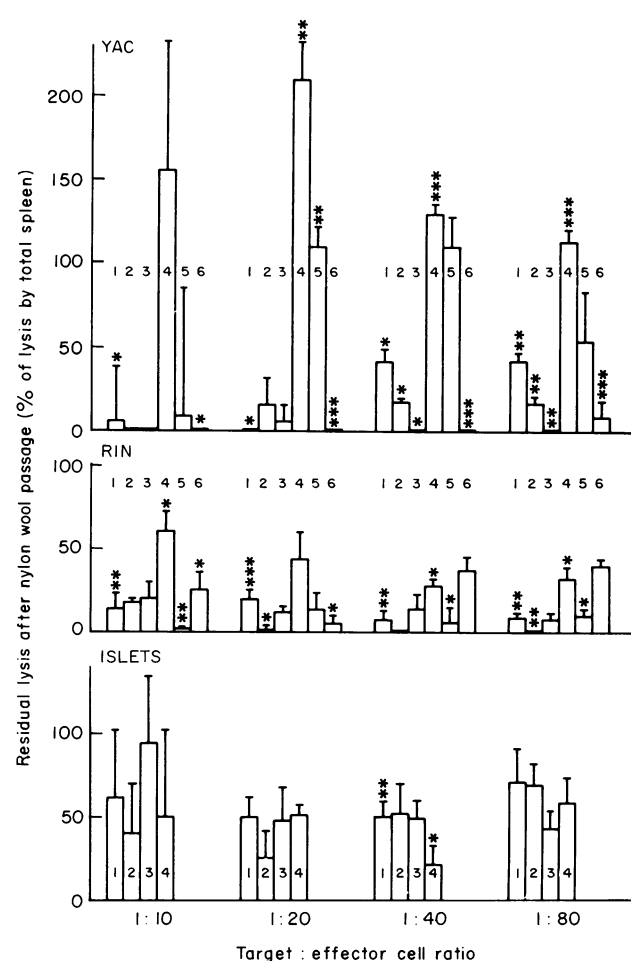
Fig. 1. Enhanced cytotoxicity by spleen cells from low dose streptozoto-cin-treated mice against RINm5F, YAC-1, EL-4 and islet cells. For a single test splenocytes from two mice were isolated and pooled at 10 days after the onset of streptozotocin treatment (Sz, ▤) or after sham treatment (C, □). Data show mean±s.e.m. from three tests for RIN cells, YAC-1 and EL-4 and 15 tests for islet cells. Significant differences between Sz and C are indicated. * P < 0·05, ** P < 0·01.

Table 1. Enrichment for spleen T- and NK-lymphocytes by nylon wool passage

	Spleen cells stained (% ± s.e.m.)	
Antibody	Unfractionated	After nylon wool passage
Thy 1	23·6 ± 3·3	55·8 ± 8·3
Lyt 2	14·6 ± 2·1	33·0 ± 1·6
L3T4	21·1 ± 2·4	40·7 ± 2·2
Asialo GM1	5·6 ± 0·6	26·7 ± 3·2
M1/70-15	13·7 ± 0·8	1·7 ± 0·7
Immunoglobulin	69·7 ± 2·0	7·7 ± 0·3

Data are from 3-9 cell separation experiments.

Fig. 2. Cytotoxic activity of spleen cell subsets from low dose streptozotocin-treated mice against tumour and islet cells. For each test splenocytes from two mice were isolated and pooled at 10 days after the onset of streptozotocin treatment. Results are given as residual lysis after nylon wool passage with lysis seen for unfractionated spleen cells set as 100%. Unfractionated spleen cells lysed YAC-1 cells to 5·8% at a T:E ratio of 1:10 up to 22·3% at a T:E ratio of 1:80, RINm5F cells to 15·2% (1:10) up to 29·0% (1:80) and islet cells to 4·6% (1:10) up to 12·1% (1:80). Columns number 1-6 represent the following effector populations: 1, nylon wool non-adherent spleen cells; 2, see col. 1—anti-Lyt-2 treated; 3, see col. 1—anti-AsGM1 treated; 4, see col. 1 plus IL-2; 5, see col. 4—anti-Lyt-2 treated; 6, see col. 4—anti-AsGM1 treated. The bars indicate mean±s.e.m. (n = 3). Significant differences between residual lysis after nylon wool passage (column 1) and lysis seen for unfractionated spleen cells (100%), between columns 2, 3 and 4 and column 1 and between columns 5 and 6 and column 4 are indicated. * P < 0·05, ** P < 0·01, *** P < 0·001.

cells. When macrophage-toxic silica particles were added to the cultures islet cytotoxicity was substantially reduced (Fig. 3).

The stimulation of macrophage activity in streptozotocin-treated mice was also evident from analysis of the capacity to produce radical oxygen intermediates (Fig. 4).

In addition to the higher activation state of macrophages after low dose streptozotocin, an increased rate of spontaneous thymidine incorporation was observed in unfractionated spleen cells (921 ct/min ± 139, n = 38 versus 611 ct/min ± 33, n = 62; P < 0·025). The proliferative response to Con A was reduced (59000 ± 6600 versus 90000 ± 6700, n = 3; P < 0·03) but was
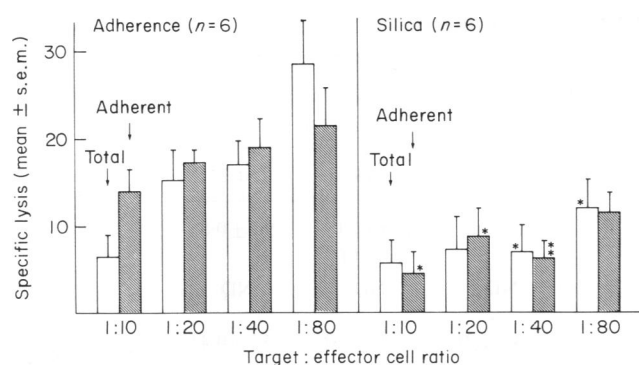
Fig. 3. Plastic adherence and silica susceptibility of effector cells in islet cytotoxicity. Splenocytes were isolated and pooled at 10 days after the onset of streptozotocin treatment. Significant differences between silica-treated and control cultures are indicated. $*P<0.05$, $**P<0.01$.
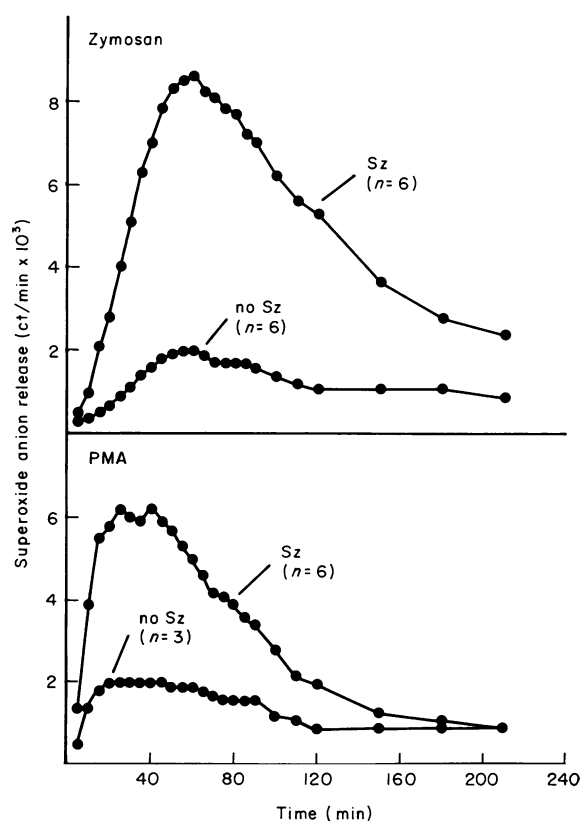

Fig. 4. Enhanced superoxide anion release by macrophages from low dose streptozotocin-treated mice. Spleen cells were isolated and adherent cells prepared at 10 days after the onset of streptozotocin treatment (Sz) or after sham treatment (C). Background levels with medium alone plus/minus phorbol myristate acetate (PMA) or zymosan were below 1000 ct/min at all points of time. Values for standard deviation were smaller than 700 ct/min and are not indicated. Maximal difference in superoxide anion release after 60 min (zymosan) or 40 min (PMA) was significant, $P<0.0001$.

increased for PHA (25000 ± 4800 *versus* 15000 ± 2400, $n=3$; $P<0.08$) and for PWM (26000 ± 4900 *versus* 10000 ± 1700, $n=3$; $P<0.004$). The number of mononuclear cells in the spleen was significantly reduced (Table 2). Furthermore a significant reduction of T-lymphocyte numbers in the peripheral blood was noted, which was almost entirely due to a loss of L3T4$^+$ T cells (Table 2).

DISCUSSION

Spleen cells showed enhanced cytotoxicity against syngeneic islet cells and various tumour cells including rat insulinoma cells when mice had been treated with multiple low doses of streptozotocin. Most of the streptozotocin-induced cytotoxic activity against tumour cells was removed by passage of spleen cells through a nylon wool column and thus probably is mediated by macrophages. The remaining lytic activity was dependent on the presence of Lyt-2$^+$ lymphocytes for RIN and on both Lyt-2 and asialo GM1$^+$ cells for YAC-1.

The effector cells involved in the lysis of islet cells were further analysed. Among adherent cells silica-susceptible macrophages were positively identified as effector cells. However, a considerable residual lysis could be detected after silica treatment of spleen cells. Furthermore, the enrichment of macrophages did not result in an increased cytotoxic activity compared with unseparated spleen cells. This may be explained by a concomitant loss of other effector cell types during the adherence step. About 50–70% of the cytotoxic activity was nylon wool non-adherent. In this cell fraction neither the removal of Lyt-2 nor of asialo GM1$^+$ cells depleted islet cell lysis, which leaves L3T4$^+$ T cells as the putative effector cell type. We conclude that multiple low doses of streptozotocin stimulate non-MHC restricted cytotoxic activity of several cell types such as macrophages, T cells and NK lymphocytes.

Several additional points should be made. RIN cells are shown to be lysed by an in part different spectrum of effector cells than normal islet cells. Thus, RIN cells are not an appropriate target when studying anti-islet immunity. McEvoy *et al.* (1984, 1987) demonstrated previously the enhanced lysis of RIN cells by spleen cells from low dose streptozotocin-treated mice. In their hands nylon wool passage enriched for cytotoxic effector cells. Unfortunately they did not analyse the cellular composition of the eluate so that a direct comparison of data is not possible.

It is not known at present whether cytotoxic lymphocytes and macrophages are directly activated through contact with streptozotocin or indirectly by immune mediators released after streptozotocin action. In favour of the latter is a report by Cockfield *et al.* (1989), presenting suggestive evidence for increased systemic IFN-γ levels after multiple low doses of streptozotocin. The increased release of IFN-γ is an additional argument against a general depression of immune reactivity after low dose streptozotocin. Finally, the analysis of spontaneous thymidine incorporation and of proliferative responses to the mitogens Con A, PHA and PWM also did not indicate a general impairment of immune functions.

In conclusion we assume that multiple low dose streptozotocin treatment affects the levels of lymphokines and monokines which results in the stimulation of 'unspecific' natural cytotoxicity. These changes in immune reactivity may promote streptozotocin-induced islet inflammation and beta cell destruction.

Table 2. Decrease of leukocyte counts after low dose streptozotocin

Treatment	Total spleen cells ($\times 10^6$, mean $\pm$ s.e.m.)	Total blood leukocytes (cells/μl, mean $\pm$ s.e.m.)	Blood leukocyte subtypes (%, mean $\pm$ s.e.m.)		
			Thy-1$^+$	L3T4$^+$	Lyt 2$^+$
Sham-treated	116 ± 20.8 ($n=40$)	13410 ± 2510 ($n=15$)	22.8 ± 2.4	15.3 ± 2.0 ($n=6$)	9.8 ± 0.8
d10 after Sz	116 ± 26.5 ($n=24$)	14410 ± 1417 ($n=5$)	ND	ND	ND
d28 after Sz	$68 \pm 28.4^*$ ($n=27$)	$10386 \pm 2985\dagger$ ($n=10$)	$13.4 \pm 2.2\dagger$	$7.9 \pm 1.6\dagger$ ($n=7$)	$7.3 \pm 0.4\dagger$

Sz, streptozotocin; ND, not done.
*Significant difference to control, $P < 0.0001$.
†Significant difference to control, $P < 0.01$.

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